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Irrigation Solutions Negatively Affect the Viability and Function of Human Fibroblasts: An in vitro Study

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Keywords

Orthopedic surgery · Cell biology · Cell viability · Fibroblast · Fibroblast function · Fibroblast viability · Physiology · Anatomy · alamarBlue reduction · Irrigation solutions · Cytotoxicity · Harmful irrigation solutions

Abstract

Introduction: Multiple irrigation solutions are used in orthopedic surgeries although there are limited studies on their lasting effects on human tissues. The purpose of this work was to investigate the cytotoxic effects of the irrigation solutions Bacitracin, Clorpactin (sodium oxychlorosene), Irrisept (0.05% chlorhexidine gluconate), and Bactisure (ethanol 1%, acetic acid 0.6%, sodium acetate 0.2%, benzalkonium chloride 0.013%, and water) on 3D cultures of human fibroblasts. ***Methods:*** Two independent experiments with 6 replicates were performed for the following conditions: Control (saline), bacitracin, Clorpactin, Irrisept, and Bactisure. Human fibroblast cell sheets were exposed to these solutions (1 or 2 min), followed by three washes with warm saline. Cell sheets were then cultured for additional 5- and 7-day posttreatment. Cell viability was measured using the alamarBlue (AB) assay. The more cytotoxic the irrigant, the lower the AB reduction. ***Results:*** For 1-min exposure time, significant differences in AB reduction were noted in Clorpactin, Irrisept, and Bactisure groups compared to control at both 5 days (Clorpactin $p = 0.0003$, Irrisept $p = 7.31 \times 10^{-15}$, Bactisure $p = 6.86 \times 10^{-14}$) and 7 days posttreatment (all groups $p < 0.0001$). The results were similar in the 2-min exposure groups. Bacitracin-treated fibroblasts displayed no significant difference at all measurement times compared to control. ***Discussion:*** Impacts of irrigation solution exposure on cell viability were varied. Irrisept and Bactisure showed the highest cell toxicity even after a brief exposure (1 min), while bacitracin and Clorpactin exposure showed smaller impacts on cell viability as compared to saline controls. This in vitro study provided insight into the effects of the irrigants on human cells and provides the groundwork essential to move to in vivo studies. Our findings raised the concern that some irrigation solutions may have negative impacts on wound healing and healthy cellular response.

Level of Evidence: Therapeutic Level V.

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Introduction

Surgical wound irrigation is commonly performed in orthopedic surgery [1] to reduce the risk of infection by flushing out bacteria [2]. When implants are present, the goal of irrigation is to prevent any retention of bacteria or biofilm on the implant surface [3]. Many irrigation solutions used clinically have different chemical makeups and concentrations, each claiming specific efficacy. While each solution may have the capability of maintaining or creating sterility, the effects of the irrigation solution on the surgical site tissues are unclear. Chlorhexidine gluconate (CHG) is a well-established irrigation solution with antimicrobial effects [4, 5]. While widely applied, CHG has been shown to have negative effects on human tissues. It prevents attachment and growth of human fibroblasts in vitro, a reliable marker for cytotoxicity and inhibition of cell regeneration [6–8]. Other in vitro studies on the effect of dosage and application time of CHG on cells including fibroblasts, endothelial cells, myoblasts, and osteoblasts demonstrated that its toxic effect was time and dose dependent [9, 10]. The structural changes of osteoblasts after exposure to CHG were described by Rohner et al. [11] as instant cell damage characterized by necrosis and apoptosis. Other authors described variable decreases in function and indicators of cell damage using various CHG concentrations (0.00009–2%) and exposure times (1 min–20 min) [12–14]. A newer CHG-based irrigation solution Irrisept® has been recently used in procedures for the beneficial antimicrobial effects, without any clinical research studying its effect on living tissues. In contrast, the antibiotic irrigation solution bacitracin has been found safe and noncytotoxic to osteoblasts [15]. Also, when used for irrigation on infected wounds after an elective orthopedic surgery, bacitracin was shown to effectively eliminate clinical infection in dogs [16]. Another seemingly less toxic and commonly used irrigation solution is Clorpactin (sodium oxychlorosene), a modified hypochlorous acid derivative [17]. Clorpactin is highly bactericidal via oxidation and hypochlorination [18] and has a relatively neutral pH (6.5–6.9). It was proven to be less irritating to human tissues than its predecessor Dakin's solution [19, 20]. The work done by Markel et al. [21] on the effect of bacitracin, Clorpactin, and Irrisept on human osteoblast cytotoxicity and proliferation showed that after exposure to these solutions for 1, 2, and 4 min all the solutions were damaging to the cells when compared to saline control. This damage was partially reversible for cells exposed to bacitracin and Clorpactin but not for the cells exposed to Irrisept. These cells showed severe signs of necrosis and death without any signs of improvement after 1 week of exposure.

Bactisure (Zimmer-Biomet, Warsaw, IN) is an acetic acid, benzalkonium chloride (BZK)-based surgical lavage solution (ethanol 1%, acetic acid 0.6%, sodium acetate 0.2%, BZK 0.013%, and water). The component BZK is a quaternary ammonium cationic detergent that acts as a surfactant and as an antibacterial agent at 0.1% concentration [22]. The manufacturer claims that the solution does not harm human tissue [22–24]. However, no outside clinical research supports this statement.

Despite the extensive clinical use of these solutions, a paucity of literature regarding specific effects of the irrigation solutions on living tissue in vivo still remains. The aim of this study was to investigate and compare the potential cytotoxic effects of the irrigation solutions: Clorpactin (United Guardian Inc., Hauppauge, NY, USA), Bactisure (Zimmer-Biomet, Warsaw, IN, USA), Irrisept (0.05% CHG in sterile water) (Irrimax Corporation, Innovation Technologies, Inc., Lawrenceville, GA, USA), and bacitracin (Sigma Aldrich) on human fibroblast in tissue-like sheets at various exposure times. Tissue-like sheets were chosen due to our findings in previous trials and past works using monolayers, where cell aggregates were found at the periphery of the culture dish. These aggregates inherently resisted the instant damage caused by some of the irrigation solutions. Additionally, cell-sheet technology has become an attractive replacement for cell monolayers for in vitro testing as it allows for deposition of extracellular matrix (ECM) and thus creates conditions more similar to an in vivo tissue microenvironment [25].

Methods

Cell Preparation

Early passage human primary dermal fibroblasts (PCS-201-012; ATCC, Manassa, VA, USA) were cultured in a 96 well plate at approximately 25,000–30,000 cells per well (80,000–100,000 cells/cm^2) to form high-density cell layers. Cells were grown in Fibroblast Basal Medium (PCS-201-030; ATCC) supplemented with Fibroblast Growth Kit-Low Serum (PCS-201-041; ATCC). Cells were incubated under 5% CO2 at 37°C for 48 h to allow for cell attachment. The medium was changed every 48–72 h for 2 weeks to promote adequate growth and the formation of multiple cell layers with ECM deposition before the application of the irrigation solutions.

Testing Conditions

The following irrigation solutions: Clorpactin, Bactisure, Irrisept, and bacitracin were obtained from hospital stores or from the manufacturers for study. Manufacturer's guidelines were used for the preparation of the solutions. We chose to use these guidelines

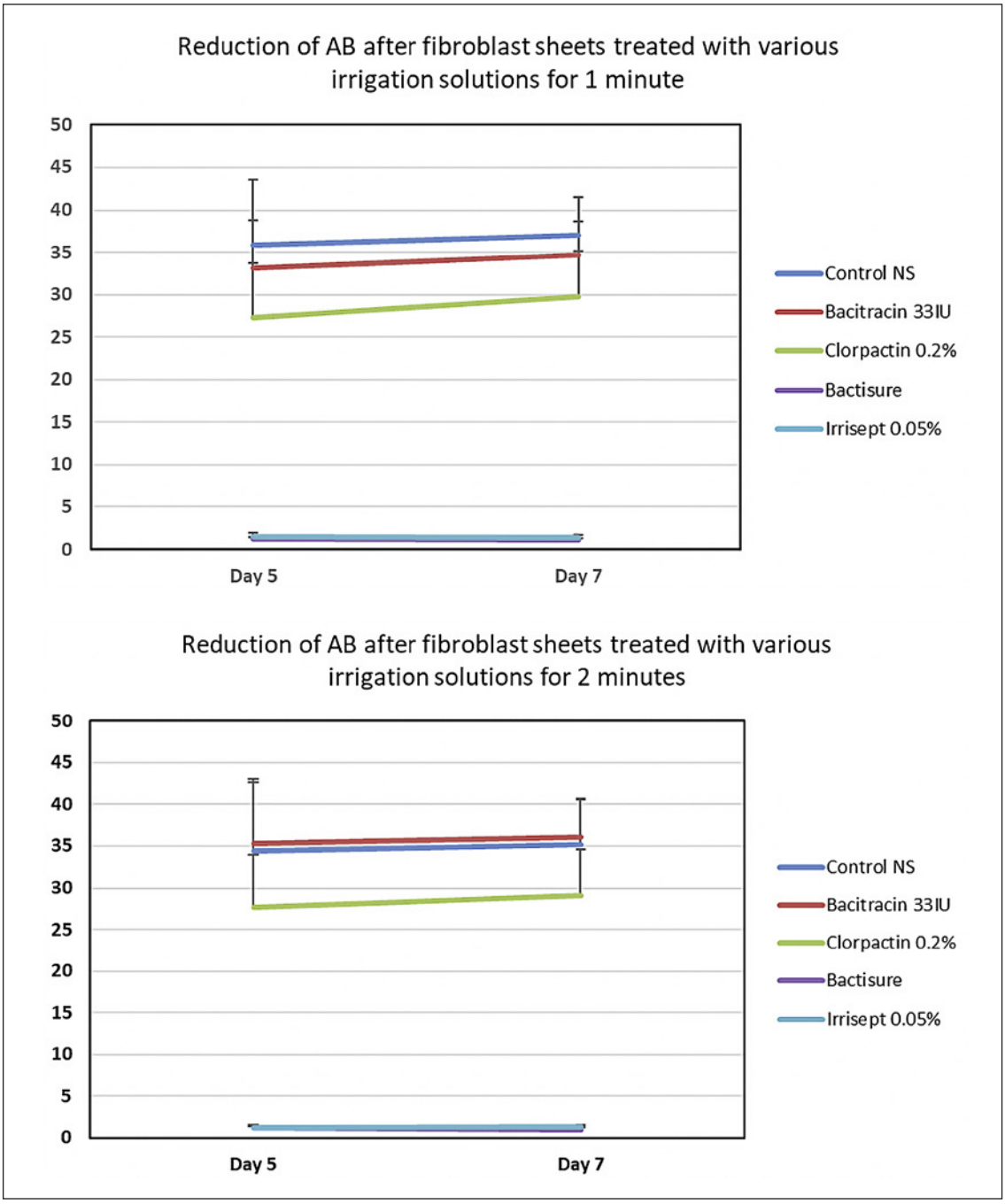

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Fig. 1. Plot representation of AB reduction at 5 and 7 days after the application of the irrigants to fibroblast sheets for 1 and 2 min.

for preparation to better replicate clinical use of these select solutions. After 14 days in culture, the cells were exposed to 100 µL of the irrigation solutions: normal (0.9%) saline, bacitracin (33 IU/mL), Clorpactin (0.2%), IrriSept (0.5% CHG), and Bactisure (0.6% acetic acid). We chose this amount and dosage of selected solutions because of the ability of 100 µL to fully cover the layered fibroblast sheets in each of the testing wells. Each solution was tested at two different exposure times: 1 min and 2 min. These exposure time points chosen were largely chosen in accordance to their clinical use, using arbitrary amounts of time in hope to sanitize and cleanse the surgical site. After exposure, the cells were washed three times with normal saline and 100 µL fresh culture medium was applied.

Table 1. AB reduction of fibroblast sheet samples

Sample	Incubation time, min	Percent reduction at day 5 (std)	*p* value compared to control	Percent reduction at day 7 (std)	*p* value compared to control
Control	1	35.88 (±7.74)	–	37.03 (±4.42)	–
Bacitracin (33 IU/mL)	1	33.15 (±5.68)	0.1813	34.64 (±4.00)	0.0701
Clorpactin (0.2%)	1	27.24 (±6.43)	0.0003	29.71 (±5.38)	<0.0001
Bactisure	1	1.30 (±0.18)	<0.0001	1.05 (±0.26)	<0.0001
Irrisept	1	1.47 (±0.25)	<0.0001	1.46 (±0.18)	<0.0001
Control	2	34.45 (±8.52)	–	35.19 (±5.35)	–
Bacitracin (33 IU/mL)	2	35.37 (±7.23)	0.7038	36.05 (±4.60)	0.5803
Clorpactin (0.2%)	2	27.67 (±6.31)	0.0064	29.05 (±5.57)	0.001
Bactisure	2	1.27 (±0.12)	<0.0001	1.02 (±0.27)	<0.0001
Irrisept	2	1.39 (±0.19)	<0.0001	1.39 (±0.19)	<0.0001

Percent reduction of alamarBlue (AB) after fibroblast sheets treated with various irrigation solutions at 1- and 2-min time points. std, standard deviation; IU, international units; mL, milliliters.

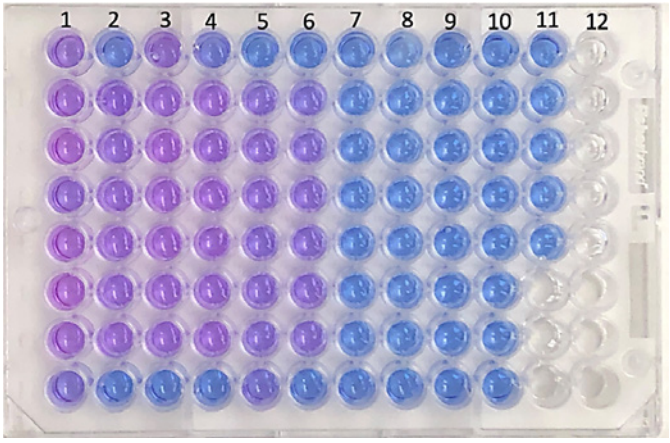

Fig. 2. AB assay sample plate after fibroblast cells incubated with AB solution for 1 h. Sample is of Trial 2: 2-min exposure, 7-day posttreatment. 1. Normal saline 1-min, 2. normal saline 2-min, 3. bacitracin (33 IU/mL) 1-min, 4. bacitracin (33 IU/mL) 2-min, 5. Clorpactin (0.2%) 1-min, 6. Clorpactin (0.2%) 2-min, 7. Bactisure (0.6% acetic acid) 1-min, 8. Bactisure (0.6% acetic acid) 2-min, 9. Irrisept (0.5% CHG) 1-min, 10. Irrisept (0.5% CHG) 2-min, 11. negative control (medium + AB only), 12. Clear (fibroblast medium only). Blue = no reduction; Red = full reduction.

Each condition and time point were tested three times, with 8 replicates for each condition. The cells were continuously cultured for 5 and 7 days, respectively, before performing the cell viability assay.

alamarBlue Assay

alamarBlue (AB) (A50101; InVitrogen, Carlsbad, CA, USA), a nontoxic, cell-permeable compound, was used to measure the viability of the cells. Living cells reduce the resazurin dye (blue) to resorufin (red). The irreversible reduction of resazurin to resorufin is mediated by intracellular diaphorase enzymes [26]. The reduction produces a fluorescent signal that is measurable via a spectrophotometer and provides a quantification of the cellular metabolic activity within a population of cells [27]. In previous studies, AB proved to be a successful agent as a noninvasive indicator to measure the cellularity within 3D ECM scaffolds [28]. In addition to the use of spectroscopy as a quantitative measure, we utilized alamarBlue to grossly visualize cell viability through color change as a qualitative assessment.

Five days after the application of the irrigation solutions to the fibroblast 3D cell sheets, medium was replaced with 100 µL fresh culture medium supplemented with 10% vol/vol AB solution. The cells were incubated under 5% CO_2 at 37°C for 1 h. After incubation with AB, 80 µL solution was transferred to a clean 96-well plate for reading and the cells were supplemented with fresh culture medium. Using a microplate reader (Synergy HT Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT, USA), the absorbance was measured at 570 nm, using 610 nm as a reference wavelength, and the percent reduction of AB was calculated per the manual's description. After another 48 h, 7-day post-irrigation solution testing on the cells, an AB assay was performed a second time to analyze whether there was a change in the cells' viability.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel. ANOVA and Paired *T* tests were used to compare the mean AB reduction of normal saline to other irrigation solutions. *T* tests were also used to compare each solution versus itself at 5 days and 7 days after testing. Analysis of variance was used to also compare the means of each irrigation solution to one another at the same reading time under the same exposure amount.

Results

The first data points were qualitative and based on the AB reduction in each treatment exposure group. The extent of AB reduction (from blue to pink) indicates the

Table 2. Fibroblast sheet samples, ANOVA comparison

ANOVA, fibroblast sheets	1-min, day 5	1-min, day 7	2-min, day 5	2-min, day 7
Control to bacitracin	0.1813	0.0701	0.7038	0.5803
Control to Clorpactin	0.0003	<0.0001	0.0064	0.001
Control to Bactisure	<0.0001	<0.0001	<0.0001	<0.0001
Control to Irrisept	<0.0001	<0.0001	<0.0001	<0.0001
Bacitracin to Clorpactin	0.0019	0.0011	0.0007	<0.0001
Bacitracin to Bactisure	<0.0001	<0.0001	<0.0001	<0.0001
Bacitracin to Irrisept	<0.0001	<0.0001	<0.0001	<0.0001
Clorpactin to Bactisure	<0.0001	<0.0001	<0.0001	<0.0001
Clorpactin to Irrisept	<0.0001	<0.0001	<0.0001	<0.0001
Bactisure to Irrisept	0.0129	<0.0001	0.0072	<0.0001

ANOVA comparison of treatment groups separated by exposure time (1-min, 2-min) and reading time following exposure (day 5, day 7).

volume of cell viability. Grossly, after the 1-h incubation time, it appeared that the AB had been reduced from blue to pink in the control and the bacitracin-treated groups, and partially reduced in the Clorpactin-treated group as it showed a purplish color. On the other hand, the Bactisure and Irrisept groups showed minimal color change when compared to the AB and media-only wells indicating poor cell viability (Fig. 1).

Treatment with bacitracin on fibroblast sheets showed no statistical difference when compared to the control, normal saline. This was evident for the 1-min and 2-min time points at both the 5-day and the 7-day posttreatment data collection times (5 days $p = 0.1813$ and 0.7038; 7 days $p = 0.0701$ and 0.5803).

As shown in Table 1, all Clorpactin-treated cells showed statistical significance of percent AB reduction when compared to the control regardless of exposure time at both 5-day (1-min exposure: $p = 0.0003$, 2-min: $p = 0.0064$) and 7-day posttreatment (1-min exposure: $p = <0.0001$, 2-min: $p = 0.0010$). Results are plotted in Figure 1.

Both Irrisept and Bactisure-treated groups demonstrated significant differences in percent AB reduction at both 5-day and 7-day posttreatment when compared to controls. Qualitatively, Figure 2 shows little to no color change in both the Irrisept and Bactisure-treated groups.

All Bactisure-treated groups showed statistical significance of percent AB reduction when compared to the control regardless of exposure time at both 5-day (1-min exposure: $p < 0.0001$, 2-min: $p = 0.0001$) and 7-day posttreatment (1-min exposure: $p < 0.0001$, 2-min: $p < 0.0001$). Little to no cell activity was noted following treatment at both 5-day and 7-day posttreatment time points, and the percent AB reduction only varied slightly when comparing the day 5 and day 7 posttreatment collection data.

Similarly, all Irrisept treated groups showed a statistical significance of percent AB reduction when compared to the control regardless of exposure time at both 5-day (1-min exposure: $p < 0.0001$, 2-min: $p = 0.0001$) and 7-day posttreatment (1-min exposure: $p < 0.0001$, 2-min: $p < 0.0001$). Little to no cell activity was noted following treatment at both 5-day and 7-day posttreatment time points and the percent AB reduction only varied slightly when comparing the day 5 and day 7 posttreatment collection data.

Using ANOVA, the AB reduction means of each group of fibroblasts exposed to the different irrigation solutions were compared to one another with the same exposure time, at the same reading time post-exposure. After 1-min exposure and 5 days after said exposure, all groups compared within said time point were statistically significant except the Control and bacitracin ($p = 0.1813$), as previously mentioned. Results were similar from the day 7 reading time after 1-min exposure (Control to bacitracin, $p = 0.0701$). Bactisure and Irrisept AB means were shown to slightly more correlation, however still significantly different. Similar results as aforementioned were found during the 2-min exposure trials at both day 5 and day 7 readings as well. Results shown in Table 2.

Discussion

Using the calculation of percent AB reduction as a predictor for cell function, bacitracin-treated fibroblasts demonstrated similar cell function to controls with dif-

ferent exposure times of 1 and 2 min. Therefore, the fibroblasts treated with bacitracin retained their viability and ability to function with respect to their ability to reduce the AB. These data suggest that bacitracin had little to no inhibitory effect on fibroblast cell function with respect to the experimental exposure times. These novel insights we have found create an interesting foreground to compare to established data previously ascertained. A past study used bacitracin and compared it to other antiseptic solutions (betadine solution, betadine scrub, and hydrogen peroxide) to test the toxicity of each one on osteoblasts. After a 2-min exposure time, the bacitracin exposed osteoblasts remained unharmed [15]. In contrast, groups treated with Clorpactin had a slightly lower ability to reduce AB. These findings were replicated in all trials with different exposure times indicating that the Clorpactin effect on the layered fibroblasts' function was mild. The *p* values shown in Table 2 further depict Clorpactin's effects on fibroblast function. However, significance was still demonstrated when compared to all other irrigation solutions at respective time points. We suspect that the effects of the Clorpactin are related to its hypotonic nature, which had been inherited to a lesser degree from its predecessor, Dakin's Solution [19, 20]. It was interesting to note that our results showing that the viability of the fibroblasts was similar in both 1- and 2-min treatment times indicating that no additional damage was caused after the 1-min exposure. Our results also show that fibroblast function remained inhibited to a similar degree even 7 days after treatment indicating that the cells need more time to recover after the treatment.

Exposure to the Irrisept or Bactisure had very significant negative effects. The fibroblasts had a significant decrease in the ability to reduce AB, indicative of severely inhibited cell function with Irrisept or Bactisure treatment regardless of exposure time. Interestingly, these two irrigation solutions appeared toxic to fibroblasts at clinical concentrations with the short exposure time of only 1 min. In previous studies, benzalkonium chloride, the surfactant in Bactisure, had been shown to be cytotoxic to articular chondrocytes when coupled with betamethasone in joint injections [29]. Others have found that acetic acid, another ingredient of Bactisure, to potentially be toxic to human tissue at higher concentrations [30]. However, with a clinical concentration of 1%, it did not show toxic effects on human tissues and retained good biofilm, eliminating potential in chronic wounds. While acetic acid at this concentration may have been shown safe, fibroblast sheeted samples treated with Bactisure, 0.6% acetic acid, were rendered nonfunctional regardless of exposure time. The other ingredients or combination thereof that makeup of Bactisure may potentiate the volatile effects of the acetic acid on the living cells.

Similar to Bactisure, fibroblast sheets treated with Irrisept, 0.5% CHG, had little to no percent AB reduction, indicating that the treatment solution was cytotoxic to fibroblasts regardless of the exposure time. CHG solution used in the musculoskeletal system has been shown to reduce functionality of fibroblasts, endothelial cells, and osteoblasts in a time and dose dependent manner [9, 31]. Those results concurred with our findings that Irrisept was cytotoxic to fibroblasts with any exposure >1 min. Liu et al. [12] also studied the effects of differing CHG dilutions on fibroblasts, myoblasts, and osteoblasts and concluded that the clinically used concentration of CHG (2%) was toxic to these cells in vitro. Same toxic effect of Irrisept on osteoblasts was shown by Markel, et al. [21]. Another past study used CHG at concentrations 5–2,400 times below those used clinically on in vitro fibroblasts as well and found that the CHG totally suppressed cell proliferation [32]. These low concentrations were matched with longer exposure times varying from 3 h to 24 h, exponentially longer than the exposure times used in our study. It is curious that with such a low concentration of CHG within Irrisept cell survival is still impaired. This begs the question as to what time of exposure or at what concentration does cytotoxicity start to develop.

Our study had some limitations. The main limitation was the in vitro study design and the use of AB as the only method of quantification. While animal trials are desired and planned, in vivo work was a requirement for our institution's Animal Care Research Committee. We believe that the in vivo work provided well-founded data and information on its own right but has also provided the groundwork for further in vitro studies with different cell lines as well as animal studies. While we would assume that the use of these irrigation solutions in vivo would yield similar outcomes, this cannot be guaranteed. In in vivo, there are different metabolic and fluidic environments and more complex response. Also, this study does not include findings to highlight the primary use of each irrigation solution; to eliminate biofilm and halt bacterial colonization. Each solution has clinically been proven to improve bacterial eradication when used in the operating room. Due to the in vitro study-design limitations, eradication of such biofilms was unattainable. These questions may be tested in future in vivo studies that study select solutions' antibacterial qualities in conjunction with the scarcely documented effects on native cell lineages highlighted in our present study. Another limitation in these

experiments was the singular cell line used, which could create a different response than the primary cells found in vivo, due to the diverse cell lineages and ECM in the surrounding bioenvironment. Lastly, the timeframe of AB measurements may have limited the measurement of potential functional recovery. It is conceivable that 7 days was insufficient to show complete recovery of the treated cells. That said, for the most part function remained similar and showed little to no change or recovery after 5 days.

Our primary outcome was to study the potential volatile effects on human fibroblasts in vitro through the use of AB to evaluate cell viability. This outcome was successfully attained and shown through our results indicating that certain irrigation solutions displayed more cytotoxicity than others. Our results display an importance that can be represented in the clinical setting, where surgeons should take into account not only the ability of select solutions to eliminate bacterial burden, but also maintain the viability of native tissue. The fact that some solutions were more volatile than others may help surgeons quantify the burden on native tissue while deciding the amount of exposure time used in the clinical realm. We hope that our findings help aid orthopedic surgeons in making important clinical decisions to help guide the preferential use of certain irrigation solutions for utmost sterility and maintenance of native cell viability.

Conclusion

Significant dysfunction of fibroblast sheeted cells was demonstrated following exposure to some commonly used perioperative irrigation solutions. These results suggest that the use of solutions such as Clorpactin or bacitracin may be less harmful to tissues and behave relatively similar to normal saline. In contrast, the lavages Bactisure and Irrisept, both created for their bactericidal biofilm effects, were shown to be extremely volatile and caused significant toxicity to fibroblasts grown in 3-dimensional sheets at clinical concentrations. It may be wise to recommend limited exposure times and/or copious posttreatment saline wash when using these materials intraoperatively to protect the underlying tissues. This in vitro study remains essential and required in preparation for in vivo studies currently underway. The concept proven in our studies should not go unrecognized and shall continue to be studied in ongoing tissue studies on the implications of exposure times of preferential surgical cleansing solutions.

Statement of Ethics

This study was carried out as an in vitro study. The IRB for Ascension Providence Hospital reviewed our study and has stated that our present study is exempted and does not require a review by an ethical committee based on the institution's IRB standards for determination of human subject research, form IRB-SOB-207.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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There was no funding of research relevant to our study.

Author Contributions

Primary author and contributor – David Sosnoski, DO. Corresponding authors – Therese Bou-akl, MD PhD; Paula Dietz; Wei-Ping Ren, MD, PhD; and David Markel, MD. Data gathering – David Sosnoski, DO, and Paula Dietz.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Any further inquiries may be directed to the corresponding author.

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